A study of host defence peptide β-defensin 3 in primates

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We have investigated the molecular evolution of the gene coding for β-defensin 3 (DEFB103) in 17 primate species including humans. Unlike the DEFB4 genes (coding for β-defensin 2) [Boniotto, Tossi, Del Pero, Sgubin, Antcheva, Santon and Masters (2003) Genes Immun. **4**, 251–257], DEFB103 shows a marked degree of conservation in humans, Great Apes and New and Old World monkeys. Only the *Hylobates concolor* defensin hcBD3 showed an amino acid variation $Arg^{17} \rightarrow Trp^{17}$ that could have a functional implication, as it disrupts an intramolecular salt bridge with Glu^{27}, which locally decreases the charge and may favour dimerization in the human congener hBD3. This is thought to involve the formation of an intermolecular salt bridge between Glu^{28} and Lys^{32} on another monomer [Schibli, Hunter, Aseyev, Starner, Wiencek, McCray, Tack and Vogel (2002) J. Biol. Chem. **277**, 8279–8289]. To test the role of dimerization in mediating biological activity, we synthesized hBD3, hcBD3 and an artificial peptide in which the Lys^{26}-Glu^{27}-Glu^{28} stretch was replaced by the equivalent Phe-Thr-Lys stretch from human β-defensin 1 and we characterized their structure and anti-microbial activity. Although the structuring and dimerization of these peptides were found to differ significantly, this did not appear to affect markedly the anti-microbial potency, the broad spectrum of activity or the insensitivity of the anti-microbial action to the salinity of the medium.

Key words: anti-bacterial, anti-microbial peptide, innate immunity.

INTRODUCTION

Human β-defensin 3 (hBD3) was originally isolated from the epidermal keratinocytes of patients with psoriasis [1]. Concurrently, the gene encoding hBD3 was independently described by two groups [2,3] based on the observation that both α- and β-defensins exist as a gene cluster on chromosome 8p22–p23. hBD3 is much more basic compared with the other three characterized human β-defensins 1, 2 and 4 [4], having a total net positive charge of +11, against 5–7 for the others. hBD3 possesses bactericidal activity against Gram-positive and -negative bacteria, including multidrug-resistant *Staphylococcus aureu*s, vancomycin-resistant *Enterococcus faecium* and reportedly also *Burkholderia cepacia*, in addition to the yeast *Candida albicans* [1,3]. Furthermore, unlike other defensins, it was demonstrated to retain its microbicidal activity against *S. aureus* also at physiological salt concentrations, demonstrating some haemolytic activity in the presence of PBS [1]. Synthetic hBD3 was also reported to form non-selective ion channels in the membranes of *Xenopus laevis* oocytes [3].

hBD3 is expressed in placenta, testis, oesophagus, heart, skeletal muscle, trachea, oral mucosa and skin [2] and, thus covers both epithelial and non-epithelial cells [3]. It appears to be strongly up-regulated in primary keratinocytes by interferon-γ [3]. The peptide was also shown to activate monocytes, exerting a moderate chemotactic activity.

Structurally, β-defensins are interesting molecules in that they present a conserved tertiary-structure scaffold, based on a triple-stranded, anti-parallel β-sheet core, in the absence of significant sequence similarity. This may be because folding depends primarily on the formation of disulphide bridges and not on hydrophobic interactions. Thus only the six cysteine residues and two other small residues, which are the only significantly buried ones, are highly conserved in all β-defensins [5]. Thus the evolutionary variation of these molecules should be relatively free of structural restraints resulting from the sequence in response to selective pressures coming from the environment in relation to their function. We have begun to study systematically these variations in the closely related primates to determine whether these could reflect on the functional roles of these molecules. In a previous study [6], we found that the DEFB1 genes, coding for β-defensin 1 congeners, seemed to show a random nucleotide variation consistent with the neutral theory of molecular evolution. The DEFB4 genes (formerly DEFB2 in humans), coding for β-defensin 2 congeners, instead show at least two points at which a positive selective pressure for variation occurred in the phylogenetic tree: at the branching of the Great Apes and humans with gibbons (Hylobatidae) and at the branching of all these primates with the Cercopithecidae (Old World monkeys) [7].

In the present study, we have investigated the molecular evolution of β-defensin 3 in primates, by comparing orthologous DEFB103 genes (formerly DEFB3 in humans) from several

Abbreviations used: CFU, colony-forming unit; dS, number of synonymous nucleotide substitutions per synonymous site; dN, number of non-synonymous nucleotide substitutions per non-synonymous site; ESI, electrospray ionization; hBD, human β-defensin; hcBD, *Hylobates concolor* β-defensin; MHB, Mueller Hinton broth; MIC, minimum inhibitory concentration; ONPG, *o*-nitrophenyl β-D-galactopyranoside; SAB, Sabouraud/dextrose; SPB, sodium phosphate buffer; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFE, trifluoroethanol; TSB, Tryptic soy broth. Primate species: *CAE*, *Cercopithecus aethiops*; *CER*, *C. erythrogaster*; *CPR*, *C. preussi*; *GGO*, *Gorilla gorilla*; *HCO*, *Hylobates concolor*; *HLA*, *H. lar*; *HMO*, *H. moloch*; *MFA*, *Macaca fascicularis*; *MMU*, *M. mulatta*; *PCR*, *Presbytis cristata*; *PME*, *P. melalophos*; *POB*, *P. obscurus*; *PPA*, *Papio anubis*; *PPY*, *Pongo pygmaeus*; *PTR*, *Pan troglodytes*; *SOE*, *Saguinus oedipus*.

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primate species, as this might give new insights regarding the relationship between structural characteristics of the protein and its function. On the basis of these results, three peptides were synthesized and experiments were performed to determine the effect of variations on structuring and how this reflected on the anti-microbial activity.

EXPERIMENTAL

Chemicals and reagents

2-Chlorotrityl chloride resin, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxybenzotriazole, benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and fluoren-9-ylmethoxycarbonyl-protected amino acids were obtained from Applied Biotech (Milan, Italy); tris-(2-carboxyethyl)-phosphine hydrochloride was from Sigma (Deisenhofen, Germany). All other reagents and solvents were of synthesis grade. Mueller Hinton broth (MHB), Tryptic soy broth (TSB) and Bacto agar were obtained from Difco Laboratories (Detroit, MI, U.S.A.); *o*-nitrophenyl β-D-galactopyranoside (ONPG) was from Sigma (St. Louis, MO, U.S.A.).

DNA sequencing

Both exons of the gene coding for β-defensin 3 congeners (the nomenclature DEFB103 is maintained for all the primates) and short portions of their untranslated flanking and intronic regions have been investigated in 16 primate species for interspecific variability. We analysed three species of Great Apes [two *Pan troglodytes* (*PTR*) individuals, one *Gorilla gorilla* (*GGO*) and three *Pongo pygmaeus* (*PPY*)], three species of the family Hylobatidae [three *Hylobates concolor* (*HCO*), two *H. lar* (*HLA*) and one *H. moloch* (*HMO*)], nine species of Cercopithecidae [15 *Macaca fascicularis* (*MFA*), five *M. mulatta* (*MMU*), three *Papio anubis* (*PPA*), 20 *Cercopithecus aethiops* (*CAE*), one *C. erythrogaster* (*CER*), one *C. preussi* (*CPR*), three *Presbytis cristata* (*PCR*), three *P. obscurus* (*POB*) and three *P. melalophos* (*PME*)] and one platyrrhine species [two *Saguinus oedipus* (*SOE*)], for a total of 68 individuals.

Genomic DNAs were extracted from the liver, bone and muscle tissues using the phenol/chloroform method [8]. DNA was extracted from hair using the Chelex 100 method [9]. Genomic amplification of orthologous DEFB103 genes was performed using the designed primers based on the published human sequence (GenBank® accession no. AF516673). For the first exon, the forward primer was 5′-CAGCCACATGCCCTGACACTAT-3′ and the reverse primer was 5′-TGGTCCAAAGCACTCTGAAGG-3′. For the second exon, the forward primer was 5′-TCTGACCCACACCCAGACAA-3′ and the reverse primer was 5′-TGGTCCAAAGCACTCTGAAGGA-3′. For both exons 45 cycles of PCR were performed using the GeneAmp PCR System 9700 (Applera Genomics, Foster City, CA, U.S.A.) with 1 unit of Taq Gold (Applera Genomics) and an annealing temperature of 55 °C. The pGEM-T Easy Vector System (Promega, Madison, WI, U.S.A.) was used for cloning of PCR products. Recombinant plasmids were propagated in DH5-α bacteria. Ten recombinants were chosen and plasmids were extracted using standard protocols [8] to exclude the possibility of obtaining sequences from paralogous genes or pseudogenes that could be present in the genome of the studied species. DNA sequencing of the p-GEM plasmids was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit v2.0 (Applera Genomics). DNA sequences were detected and analysed on an automated ABI Prism 3100 Genetic Analyser (Applera Genomics).

Multiple alignments of the nucleotide and amino acid sequences were performed using the CLUSTAL X program [10]. Pairwise comparisons between nucleotide sequences were performed to investigate the extent of evolutionary constraint at the amino acid level in the molecule. The number of synonymous nucleotide substitutions per synonymous site (dS) and the number of non-synonymous nucleotide substitutions per non-synonymous site (dN) between each pair of orthologous sequences were computed by the method of Nei and Gojobori [11], using the Mega 2.0 software [12].

Structure and sequence analysis

The structure of hBD3 was obtained from the Protein Data Bank (entries 1KJ5 and 1KJ6) [13] and analysed using WebLab ViewerPro v4.0 (Molecular Simulations Inc., San Diego, CA, U.S.A.). The sequences of hBD3 and hcBD3 (HCO BD3) were analysed in terms of the positional residue frequencies for 66 β-defensin sequences obtained from the anti-microbial sequence database AMSDb (http://www.bbcm.units.it/~tossi/), using the conserved cysteine residues for correct alignment. The reported fragments from the second exon of 28 putative new hBD genes and 43 new mouse β-defensin genes were obtained from [21] (see Table 1 in Supplementary material there). This allowed an estimation of residue conservation at key positions, in particular, for the known defensin sequences, and identification of a putative, closely related mouse homologue.

Peptide synthesis

Solid-phase peptide syntheses were performed on a PE Biosystems Pioneer® peptide synthesis system (Foster City, CA, U.S.A.) with the column thermostatically maintained at 50 °C and loaded with 2-chlorotrityl chloride resin (substitution of 0.27 mmol/g of resin with lysine). Insertion of the C-terminal amino acid was performed manually according to the instructions provided in the synthesis notes section (section 2.17) of the 2003 Novabiochem catalogue. A 4-fold excess of fluoren-9-ylmethoxycarbonyl amino acid/TBTU/di-isopropylethylamine (1:1:1.7, by vol.) and a version of the anti-aggregation solvent 'magic mixture' [dimethylformamide/*N*-methylpyrrolidone (3:1, v/v), 1 % Triton X-100 and 1 M ethylene carbonate] were used for each coupling step. Double coupling with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate or benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate instead of TBTU was performed at sections predicted to be difficult by the Peptide Companion software (CoshiSoft, AZ, U.S.A.). The synthesis was interrupted regularly to check synthesis quality by MS [electrospray ionization (ESI)–MS; Perkin Elmer/Sciex API I, PE Biosystems, Foster City, CA, U.S.A.]. Peptides were then cleaved from the resin and deprotected using a cocktail consisting of trifluoroacetic acid, water, thioanisole, phenol, ethanedithiol and tri-isopropylsilane mixture (82.5:5:5:2.5:2.5:2.5, by vol.).

The crude peptides were reduced with 10-fold excess of tris-(2-carboxyethyl)-phosphine hydrochloride in 6 M guanidinium chloride/0.1 M sodium citrate buffer (pH 3.0) for 60 min at 60 °C, and immediately desalted on a preparative C_{18} RP-HPLC column [Waters Delta-Pak® C_{18}, 15 μm, 300 Å (1 Å = 0.1 nm), 25 mm × 100 mm] using N_2-saturated solvents. The fraction containing the reduced peptide, as determined by MS analysis was oxidatively folded for 24–48 h in N_2-saturated aqueous buffer

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(1 M guanidinium chloride/0.1 M ammonium acetate/2 mM EDTA, pH 8.5), in which 1 mM cysteine and 0.1 mM cystine were dissolved immediately before use, at a final peptide concentration of 10 μM. Oxidation was monitored by analytical RP-HPLC [Waters Symmetry® C_{18}, 3.5 μm, 100 Å, 4.6 mm × 50 mm] and ESI–MS until completion before final desalting and purification on the preparative column. Peptide concentrations were determined based on the molar absorption coefficients ε_{280} of tryptophan, tyrosine and cysteine residues, using the ProtParam tool on the Expasy server (http://www.expasy.ch/tools/protparam.html). They were verified using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.).

CD

Structuring was probed by CD spectroscopy on a Jasco J-715 spectropolarimeter (JASCO Corporation, Hachioji City, Tokyo, Japan), using 2-mm path-length quartz cells and peptide concentrations of 20 μM, in 5 mM sodium phosphate buffer (SPB) at pH 7.0, in the presence or absence of 50 % (v/v) trifluoroethanol (TFE) and SDS micelles (10 mM SDS in buffer). Spectra are the average for at least two independent experiments, each with accumulation of three scans.

PAGE

Electrophoresis of 3 μg each of hBD3, hcBD3 and ftkBD3 (an artificial chimaeric molecule; see the Results and discussion section for details) was performed under reducing (with 2-mercaptoethanol) and non-reducing (without 2-mercaptoethanol) conditions, in the absence of urea. Peptide stock solution (3 μl) was suspended in 5 μl of 2 × Laemmli sample buffer [4 % (w/v) SDS; Sigma], with or without 2-mercaptoethanol (10 %, v/v), taken to a final volume of 10 μl, heated to 100 °C for 5 min and loaded on to a standard SDS/polyacrylamide gel (20 %, w/v). To optimize separation, different cathode (0.1 M Tris/0.1 M tricine/0.1 % SDS, pH 8.25) and anode (0.2 M Tris, pH 8.9) buffers were used. The gel was stained with Coomassie Blue dye.

Anti-microbial activity

The anti-microbial activity of the synthetic peptides was determined against *Escherichia coli* ML-35, *Pseudomonas aeruginosa* A.T.C.C. 27853, *B. cepacia* 6981 and 14273, *S. aureus* 710A and a *C. albicans* clinical isolate with the minimum inhibitory concentration (MIC) using a micro-dilution susceptibility test as described previously [14,15]. Briefly, serial 1:1 dilutions of each peptide were made in 96-well microtitre plates, to a final volume of 100 μl of TSB [5 % (v/v) in 10 mM phosphate buffer/SPB, pH 7.4] or MHB [50 % (v/v) in SPB] with 1×10^5 colony-forming unit (CFU)/ml bacteria in exponential phase. Plates were incubated at 37 °C overnight and the MIC was considered as the first well without visible growth. For anti-fungal assays, fungi were cultivated on solid Sabouraud/dextrose (SAB) agar medium, and MIC values were determined in SAB medium (both 5 or 50 % in SPB).

To determine the salt sensitivity of the anti-microbial activity, 1×10^5 CFU/ml bacteria were incubated in 100 μl of 5 % MHB in SPB, in the presence of 8 μM peptide at NaCl concentrations of 0, 25, 50, 100 and 150 mM. After 3 h incubation at 37 °C, the suspension was diluted 100-fold and an aliquot plated to allow colony counts.

The kinetics of bacterial killing for hBD3 and hcBD3 were confirmed against both *E. coli* ML-35 and *S. aureus* 710A in the exponential phase (approx. 1×10^7 CFU/ml in SPB incubated at 37 °C). At different times, 50 μl of the bacteria/peptide suspension was diluted severalfold in ice-cold SPB, thus effectively blocking any further activity by the peptides, and the solution was plated on nutrient agar and incubated overnight to allow colony counts.

Permeabilization assays

Permeabilization of the cytoplasmic and/or outer membranes of *E. coli* by the peptides was evaluated by following the unmasking of cytoplasmic β-galactosidase activity using extracellular ONPG (1.5 mM in SPB) as described previously [14,15]. For these experiments, the β-galactosidase-constitutive, lactose permease-deficient *E. coli* ML-35 pYC strain was used (approx. 1×10^7 CFU/ml), exposed to a 10 μM peptide concentration. A strongly membranolytic α-helical peptide was used as a positive control [16]. Permeabilization of the cytoplasmic membrane of *S. aureus* was instead evaluated using the 6-phospho-β-galactosidase-constitutive 710A strain by the same method, but using ONPG-6P (1.5 mM in SPB) as a substrate, prepared by reacting ONPG with phosphorus oxychloride in trimethyl phosphate.

RESULTS AND DISCUSSION

The alignment of nucleotide sequences of the primate β-defensin 3-coding region is shown in Figure 1. We have calculated dS and dN by pairwise comparison. The low values of dS and dN observed are consistent with the high degree of conservation; also, dS values were always higher than dN (results not shown).

The deduced amino acid sequences for the primate species are reported in Figure 2, which confirm a marked conservation of the peptide sequence for all primates. Humans and Great Apes showed identical amino acid sequences, with the exception of a conservative $Ser^{22} \rightarrow Thr^{22}$ substitution in the mature peptide region seen only in *PTR*. The Hylobatidae sequences presented some minor variations with respect to hBD3. Three amino acid substitutions ($Ile^2 \rightarrow Leu^2$, $Ile^3 \rightarrow Met^3$ and $Arg^{17} \rightarrow Trp^{17}$) were observed in the N-terminal region of the active peptide from *HCO*. The three species of Asian leaf eater monkeys (genus *Presbytis*) showed only a conservative $Val^{20} \rightarrow Leu^{20}$ variation in the mature peptide region. The Old World monkey β-defensin 3 (Cercopithecidae) sequences were identical with hBD3, as also those for the New World monkey *SOE*. Some minor amino acid variations were observed in the β-defensin 3 pre-propeptide region, scattered among the non-human primates as shown in Figure 2.

The quite marked conservation of DEFB103 in primates was also confirmed by an analysis of several individuals belonging to each species. In fact, we sequenced (a forward and a reverse strand and another forward or reverse strand) two *PTR* individuals, three *PPY*, three *HCO*, two *HLA*, 15 *MFA*, five *MMU*, three *PPA*, 20 *CAE*, three *PCR*, three *POB*, three *PME* and two *SOE*.

To assess the functional implications of the observed amino acid changes in primate defensins, the few affected positions were analysed in terms of the residue variation observed in known defensin sequences {17 each of primate β-defensin 1 [6] and β-defensin 2 sequences [7], 14 bovid, one pig, nine rodent and eight avian sequences obtained from the AMSDb anti-microbial sequences database (see above) for a total of 66 sequences}. The β-defensin 3 sequence, apart from the conserved cysteine residues, also maintains only a few other residues that are quite conserved in defensins in general, such as Gly^{15}-Gly^{16} and Gln^{29}-Ile^{30}-Gly^{31} (hBD3 numbering; see Figure 3). Otherwise the sequence is

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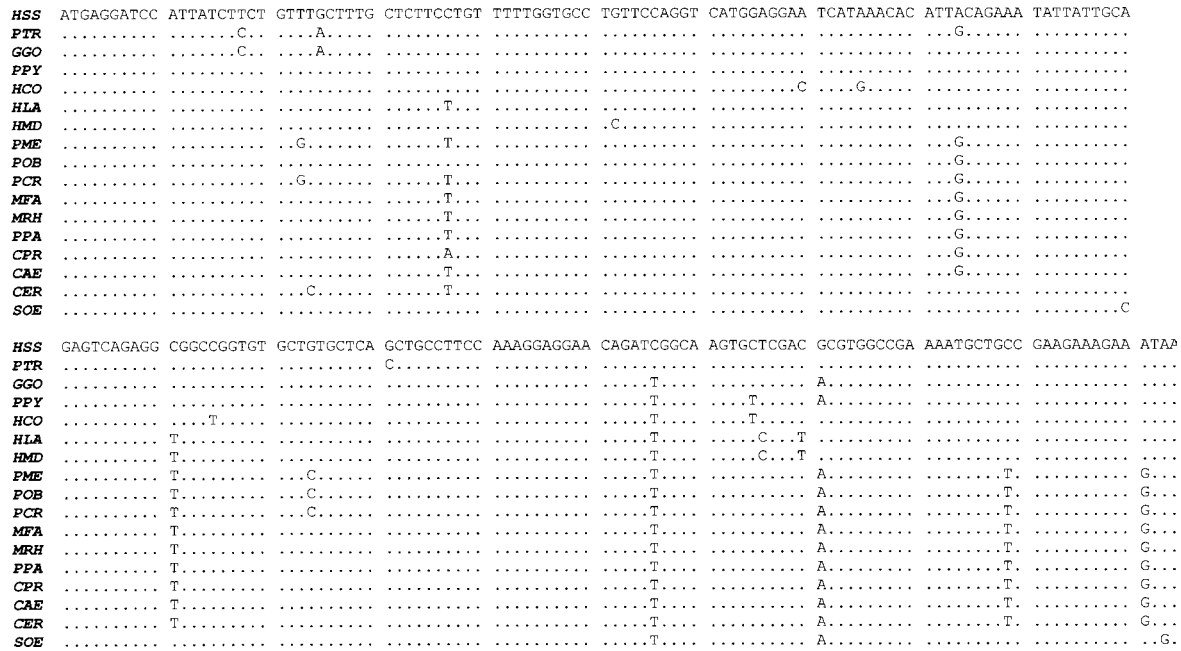

Figure 1 Alignment of nucleotide sequences from the coding region of orthologous primate β-defensin 3 genes

Dots indicate identity with human sequence (*HSS*). The following primates were analysed: *PTR*, *GGO* and *PPY* of Great Apes; *HCO*, *HLA* and *HMO* of Hylobatidae; *MFA*, *MMU*, *PPA*, *CAE*, *CER*, *CPR*, *PCR*, *POB* and *PME* of Cercopithecidae; and *SOE* of Platyrrhine species.

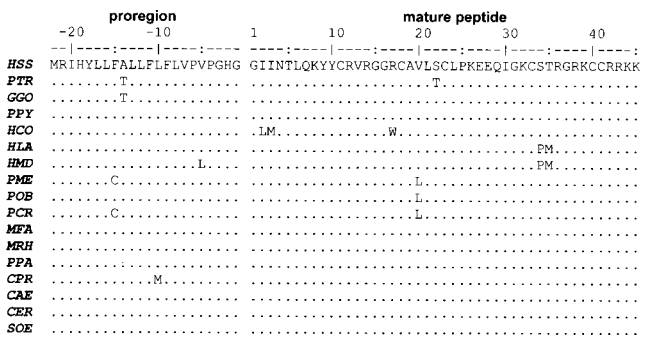

Figure 2 Alignment of the deduced amino acid sequences for primate β-defensin 3

For species names, see the abbreviation list. Dots indicate identity with the human sequence *HSS*.

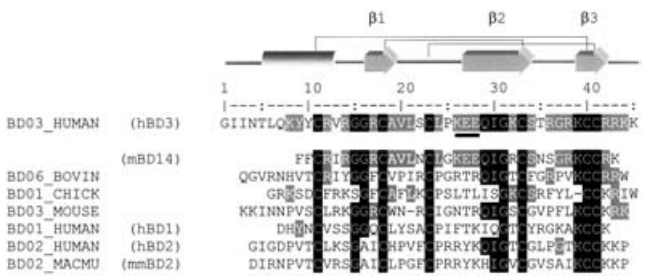

Figure 3 Comparison of the hBD3 sequence with other human, mouse, bovine or avian β-defensin sequences

Residues shaded in black are highly conserved in β-defensins in general, whereas those shaded in grey are present in a fragment of mBD14, deduced from the mouse BDEF14 gene (from [21], see Table 1 in Supplementary material there), and/or with lower frequency in other reported defensins. The numbering scheme and the topological diagram are for hBD3. Residues marked with an underline are replaced by the equivalent hBD1 residues in the synthetic peptide ftkBD3. Nomenclature shown on the left-hand side is based on AMSDb (www.bbcm.units.it/~tossi/).

quite different from that of other β-defensins. A low level of homology is observed with bovine β-defensin 6 and avian β-defensin 1 (gallinacin) and it is still less marked with other known β-defensin sequences, including hBD1 and hBD2. Sequences of the second exons from 28 putative new hBD genes and 43 new mouse β-defensin genes have been reported recently, identified by performing a bioinformatics analysis of the respective genomes [17]. A comparison of the hBD3 sequence with these indicates a considerable degree of homology with the reported fragment deriving from the mouse BDEF14 gene (see mBD14 in Figure 3). If this were confirmed to be a functional gene, it would signify that the marked degree of conservation of the β-defensin 3 sequence stretches back in evolution to rodents.

The NMR structure of monomeric hBD3 has been determined [13], and it shows the characteristic three-stranded β-sheet core with a partly helical segment at the N-terminus, as also observed in other human and mouse defensins. However, the surface of hBD3 is considerably more basic. It is suggested that this molecule can form a stable dimer through residues Glu^{27}-Glu^{28} on strand $\beta 2$ (see Figure 3), in which Glu^{28} forms a salt bridge with Lys^{32} on another monomer and vice versa, and dimerization is aided by a local decrease in charge due to intramolecular salt bridging involving Glu^{27} and Arg^{17}. It has been suggested that although these features are important to determine the potent bactericidal activity of hBD3 towards *S. aureus*, studies in which the key residues involved in dimerization are mutated were required to confirm this [13].

In this context, the only possibly significant variation observed in the primate β-defensin 3 sequences is the $Arg^{17} \rightarrow Trp^{17}$ substitution in hcBD3. To test whether the incapacity of hcBD3 to form the intramolecular salt bridge can make a difference in

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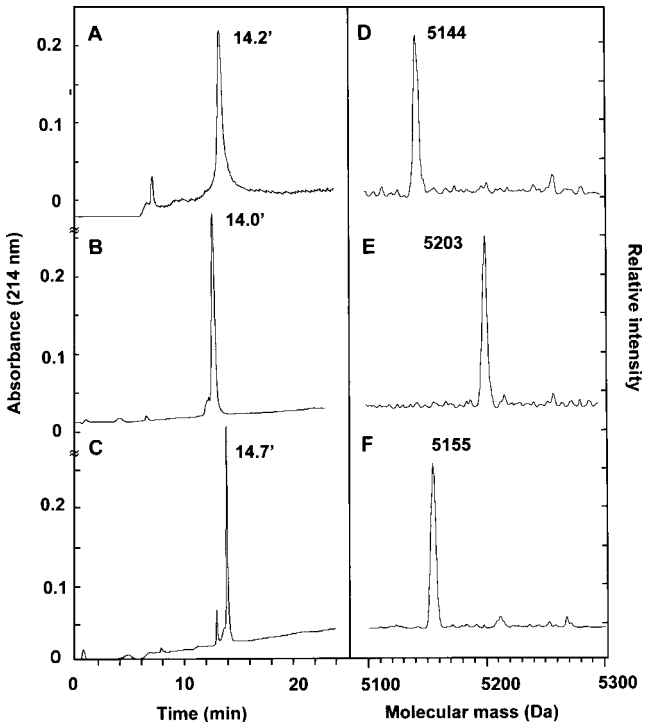

Figure 4 Analytical HPLC and ESI–MS of synthetic β-defensin 3 peptides

RP-HPLC of folded ftkBD3 (**A**), hcBD3 (**B**) and hBD3 (**C**) and the corresponding mass spectra reconstructed by the Fenn method (**D**, **E** and **F** respectively) are shown. Theoretical molecular masses of the three peptides are: hBD3, 5.1552 kDa; hcBD3, 5.2033 kDa; ftkBD3, 5.1453 kDa.

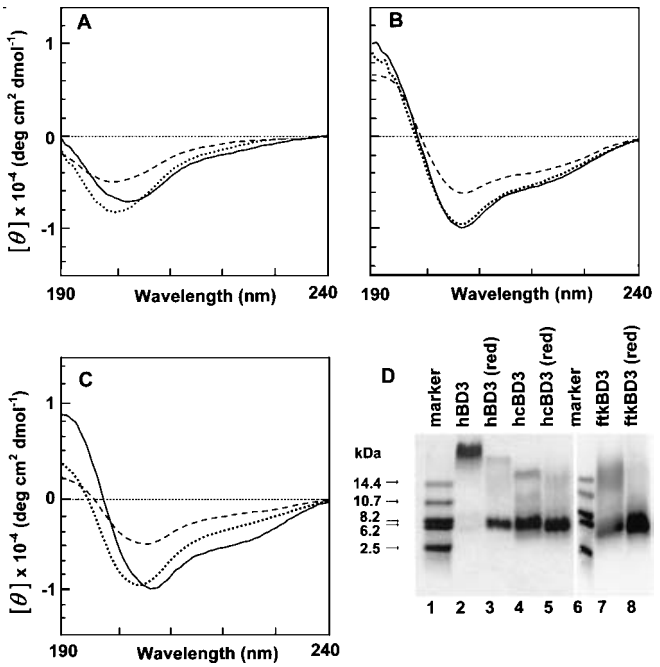

Figure 5 CD and PAGE of synthetic β-defensin 3 peptides

CD spectra of hBD3 (——), hcBD3 (·····) and ftkBD3 (----) in the presence of aqueous buffer (**A**), 50% TFE (**B**) and SDS micelles (**C**); the peptide concentration is 20 μM. (**D**) SDS/PAGE of the peptides in non-reducing (lanes 2, 4 and 7) and reducing (lanes 3, 5 and 8) conditions. Peptide markers are also shown (lanes 1 and 6).

its anti-microbial activity with respect to hBD3, it was decided to synthesize chemically and characterize both peptides. Furthermore, an artificial chimaeric molecule was also prepared in which the Lys^{26}-Glu^{27}-Glu^{28} sequence was replaced by the equivalent sequence from hBD1, namely Phe-Thr-Lys (see Figure 3). It is termed as ftkBD3. This molecule can form neither the intramolecular nor intermolecular salt bridges. Synthesis and oxidative folding of these molecules was relatively straightforward and resulted in high-quality material as indicated by analytical HPLC and MS (Figure 4). The sequence variations are accompanied by a variation in charge from +11 in the human peptide, to +10 in the *Hylobates* and +13 in the chimaeric peptides respectively.

An analysis of the structural characteristics was performed by CD spectrometry in an aqueous buffer and in the presence of the organic modifier TFE or membrane mimetic SDS micelles, as well as by native PAGE in reducing and non-reducing conditions (Figure 5). The CD spectrum for hBD3 in aqueous solution (Figure 5A) was analysed using the DICROPROT 2000 program (release 1.0.4, http://dicroprot-pbil.ibcp.fr/), which integrates into a single package with several methods designed to estimate protein secondary structure from CD results [18]. Results indicated approx. 25% of residues with a β-sheet conformation and $<10\%$ α-helical conformation in aqueous buffer, the rest being random coil. This is consistent with the presence of the β-sheet core reported for the NMR structure by Schibli et al. [13], whereas the N-terminus would appear to be unstructured under these conditions. The spectrum of hcBD3 is effectively analogous, also taking into consideration the presence of a tryptophan residue, whereas that of the chimaeric ftkBD3 seems to have a somewhat less stable structure. In the presence of 50% TFE, both hBD3 and hcBD3 undergo a marked conformational transition; whereas the β-sheet content remains the same, that of α-helix increases to approx. 25%. The N-terminus can thus assume a helical conformation in the presence of this organic modifier, which is known to stabilize this type of conformation. However, this may depend to some extent on salt bridging, as the effect on ftkBD3 is mild (Figure 5B). This transition also occurs in the presence of SDS micelles, which better mimic the anisotropic lipidic environment of membranes, but in this case to a somewhat lesser extent for hcBD3 than hBD3 (Figure 5C). This result seems to support a significant role of salt bridging both in stabilizing the structure of hBD3 and in mediating its interaction with a lipidic environment.

The effect of sequence variations on dimerization is quite evident by native PAGE (Figure 5D), where hBD3 is clearly seen to migrate more slowly than expected for the monomer, under non-reducing conditions. Indeed, the position of the band would indicate a considerably higher mass than that of a dimer. There are two possible explanations for this: (i) the compact and highly cationic (+22) dimer structure is incompletely shielded by the anionic SDS under these conditions, resulting in an apparently higher mass; and (ii) the molecule forms higher-order stable oligomers. In this respect, studies using dynamic and static light scattering seem to indicate that the dimer forms preferentially [13]. On the other hand, hcBD3 and ftkBD3 are mainly in the monomeric form also under non-reducing conditions, although a residual capacity to dimerize is apparent. In these cases, the dimeric forms (charged +20 and +26 respectively), apart from being considerably less populated, move at an apparent mass closer to the expected mass of the dimer. This could indicate either a looser structure that is better shielded by SDS or lack of higher-order oligomerization. In general, our results are in line with

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Table 1 Antimicrobial activity of β-defensins

Antimicrobial activity was tested against *E. coli* ML-35, *S. aureus* 710A, *P. aeruginosa* ATCC 27853, *B. cepacia* 6981 (a) and 14273 (b) and a *C. albicans* clinical isolate, in low concentration media [5 % v/v Tryptic soy broth (TSB) or Sabouraud broth (SAB) in 10 mM phosphate buffer] and high concentration media [50 % v/v SAB or Mueller Hinton broth (MH) in 10 mM phosphate buffer]. MIC evaluations are the mean of at least three experiments carried out in duplicate.

		MIC (μM)									
	Micro-organism . . .	*E. coli*		*P. aeruginosa*		*B. cepacia* (a)	(b)	*S. aureus*		*C. albicans*	
β-Defensin	Medium . . .	5 % TSB	50 % MH	5 % TSB	50 % MH	5 % TSB	5 % TSB	5 % TSB	50 % MH	5 % SAB	50 % SAB
hBD3		1	32	2	32	> 32	> 32	1	> 32	2	32
hcBD3		1	16	2	32	> 32	> 32	2	> 32	1	16
ftkBD3		8	8	4	> 32	> 32	> 32	4	> 32	4	16

those of Schibli et al. [13] who propose that hBD3 dimerization is mediated by an intermolecular bridge involving Glu[28]. They further indicate that the intramolecular salt bridge between Arg[17] and Glu[27] is also important for dimerization, possibly by locally decreasing the charge. This latter feature seems to have been lost in the *HCO* peptide.

Given the above results, the anti-microbial potency of the three defensins was determined against selected micro-organisms, including pulmonary pathogens, to see if it was affected by the different degrees of dimerization. MIC values were measured under both low-concentration media [5 % (v/v) TSB or 5 % (v/v) SAB] and under more robust conditions [50 % (v/v) MHB or 50 % SAB] (Table 1). Both the human and *HCO* peptides were potent and broad-spectrum antibiotics in the low-concentration medium, and the chimaeric peptide ftkBD3 also showed a somewhat decreased but still measurable activity. There are some discrepancies in the literature with respect to the Gram-negative bacterium *B. cepacia* [3,19]. In the latter extensive analysis, all the 23 tested strains of this micro-organism were resistant to hBD3 up to 100 μg/ml (equivalent to approx. 20 μM). Both the strains tested in the present study were also resistant to our peptides up to 32 μM, although a certain delay in growth was observed, consistent with a possible partial bacteriostatic activity. The anti-microbial activity was generally decreased for all micro-organisms tested in high-concentration medium, where ftkBD3, however, seemed to be less affected than the other two peptides against *E. coli* and *C. albicans* (Table 1). Under these conditions, it would thus appear that the increase in overall charge has a more relevant effect than dimerization.

To determine whether dimerization affects the salt sensitivity of the peptides, the same microbes were exposed in low-concentration medium to 8 μM peptide in the presence of increasing concentrations of NaCl (Figure 6). These assays were performed at a considerably lower peptide concentration than that reported previously for hBD3 against *S. aureus* [1] and with a wider panel of micro-organisms. Only for *E. coli* was there a significant decrease at 100 mM salt. However, hcBD3 and ftkBD3 also showed a generally unaltered activity at high salt, albeit with a slightly different pattern compared with the human peptide. Thus our results confirm and extend the potent activity of hBD3 at physiological salt concentrations. However, they also indicate a less significant role of dimerization in mediating this activity than has been proposed [13] and possibly a more significant effect of the overall charge.

To test whether the peptides act by a membranolytic mechanism, hydrolysis of the impermeant substrates ONPG and ONPG-6P were measured by unmasking cytoplasmic β-galactosidase activity in the *E. coli* ML-35 pYC and *S. aureus* 710A strains respectively, which constitutively produce these enzymes [14,15]. As can be understood from Figure 7, all three peptides seem to be capable of permeabilizing the cytoplasmic membrane of *E. coli*, although with somewhat different kinetic profiles and with a velocity that is lower than that of a reference membranolytic α-helical peptide. For *S. aureus*, measurements

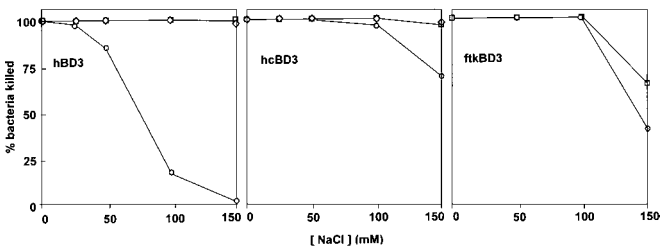

Figure 6 Salt sensitivity of anti-microbial activity

Bacterial killing was determined in terms of surviving CFUs with respect to untreated bacteria, after exposure of 1 × 10[5] CFU/ml *S. aureus* (□), *E. coli* (○) and *P. aeruginosa* (◇) to 8 μM peptide in the presence of increasing concentrations of NaCl. Results are the means for at least two independent evaluations.

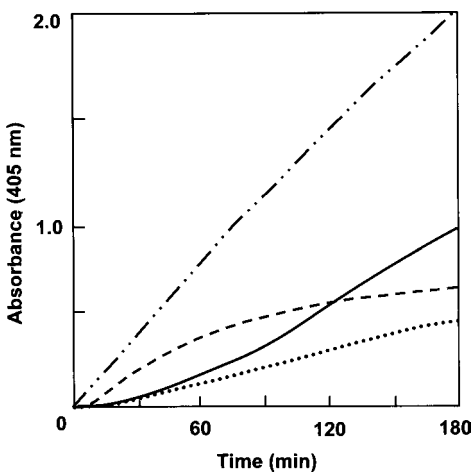

Figure 7 *E. coli* cytoplasmic membrane-permeabilization kinetics

Permeabilization was evaluated by following the unmasking of ONPG to cytoplasmic β-galactosidase, for hBD3 (——), hcBD3 (· · · · · ·) and ftkBD3 (- - - -). A permeabilization curve for the strongly membranolytic α-helical peptide P19(5|B) (-·-·-·-·-·-) is shown for comparison [16].

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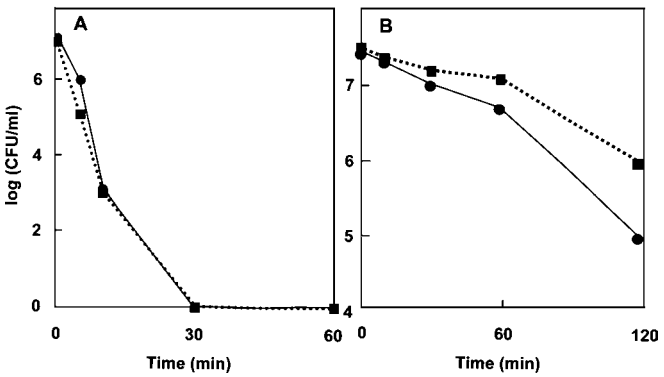

Figure 8 Kinetics of bacterial killing

Bacterial killing as a function of time for *E. coli* (**A**) and *S. aureus* (**B**) by hBD3 (—●—) and hcBD3 (· · · ■ · · ·).

were complicated by a rapid interaction of the anionic 6-phospho-ONPG with the defensin molecules, leading to a rapid colour increase followed by bleaching (results not shown). However, comparison of traces in the presence and absence of the bacterium after bleaching indicated that little permeabilization occurred in the 2-h time scale of the experiment, and results beyond this time were rendered uncertain due to instability of the bacterium at longer times under the conditions used. Thus it can be inferred that the permeabilization of the cytoplasmic membrane is much slower for the Gram-positive micro-organism.

Finally, the kinetics of bacterial killing were determined for hBD3 and hcBD3, under low-salt conditions, for both *E. coli* and *S. aureus* (Figure 8). The results were quite similar for the two defensins and, in this case, were also considerably faster for the Gram-negative bacterium. This difference in killing kinetics may be due to the particular make-up of the microbial cytoplasmic membranes, or may indicate that the outer membrane and the thin periplasmic peptidoglycan layer of *E. coli* are less of a barrier for the defensin molecules than the thicker peptidoglycan layer of *S. aureus*. A similar behaviour has been observed for membranolytic α-helical peptides [14–16,20].

Our results lead to some interesting considerations regarding the relationship between structure and activity in β-defensin 3. We have shown that relatively minor changes in the β-defensin amino acid sequence can result in considerable changes in the structure, in particular at the quaternary level, confirming an important role of intermolecular and intramolecular bridging. However, we have also demonstrated that these variations do not reflect to any significant extent on the spectrum and potency of the anti-microbial activity, its sensitivity to salt concentration or its capacity to permeabilize bacterial membranes. In fact, it has been demonstrated with other defensins that some form of direct anti-microbial activity *in vitro* can survive even more drastic structural changes, such as fragmentation or the knocking out of cystine bridges [21,22]. However, it cannot be ruled out that under physiological conditions, where interactions with host components and other molecular or cellular mediators of host defence also have to be taken into account, structural requirements are more stringent than under assay conditions *in vitro*.

In a recent study on the evolution of the β-defensin genes, Semple et al. [23] demonstrated by a computational approach, followed by expression studies, that the 8p22–p23 defensin locus in mammals evolved by successive rounds of duplication followed by substantial divergence, producing a cluster of paralogous genes defined by the four known β-defensin genes, four novel genes and a related pseudogene. The authors also reported that the divergence was most rapid within the second exons of these genes, which encode the mature β-defensin peptide, with many comparisons between paralogous genes showing an excess of non-synonymous over synonymous substitutions. The principal parameter underlying this variation seems to be the charge. In this respect, hBD3 is unusual in that it seems to be quite conserved with respect to rodents and also has an exceptionally high charge compared with the other gene products.

In conclusion, our results, while confirming the specific dimerization mode of hBD3 and also its particular anti-microbial properties *in vitro*, do not find in these activities an adequate explanation for the marked conservation of the DEFB103 gene in primates. In particular, the chimaeric peptide ftkBD3 appears to maintain a measurable activity, even in a high-salt medium, despite both inter- and intramolecular salt bridges being knocked out. It thus remains to be determined what mechanism underlies the marked conservation of DEFB103, in contrast with the considerable variation observed for other defensins and in particular for the DEFB4 gene in primates, which appears to have resulted from positive selection [7]. This could be related to its maintenance of a high charge, affecting in particular the anti-microbial activity under *in vivo* conditions, or to roles of the peptide in host defence, such as for example chemotactic activity towards monocytes, or possibly to other roles which are yet to be defined. Useful insights may come from studies on polymorphisms in human peptides and their effects on host defence.

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